Comparison of Epithelium Permeability between *in vitro* organotypic, Ex-vivo and Explant Foreskin Models and Feasibility for Co-Culture with Bacteria

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Background

The penile microbiome: an emerging HIV risk factor

- The foreskin- a major site of HIV viral entry- creates a unique microenvironment conducive to colonization by anaerobic bacteria.[1-3]
- Abundance of **penile anaerobes** has been associated with increased **local inflammation** and **risk of HIV** acquisition.[4]
- Six anaerobic bacterial species have been identified that uniquely correlate with increased risk of HIV seroconversion, increased local production of inflammatory cytokines, and increased tissue T cell density in the inner foreskin. This group has been named **Bacteria Associated with HIV Seroconversion, Inflammation and Immune Cells (BASIC).**[5]
- These findings strongly suggest that specific penile anaerobes cause inflammation in the foreskin, generating an environment with heightened susceptibility to HIV infection via sexual transmission.

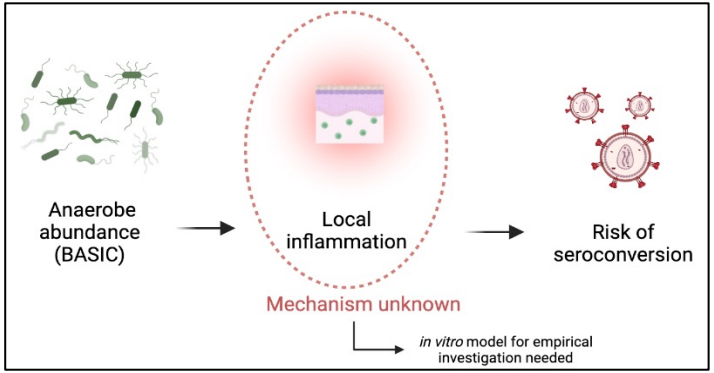

Figure 1. Overarching hypothesis. High anaerobe abundance in the penile microbiome may generate inflammation and lead to higher HIV risk, however observational data cannot identify the mechanisms by which this occurs.

The need for an organotypic *in vitro* model:

- Co-occurrence of BASIC species *in vivo* makes it nearly impossible to determine which species drive inflammation and HIV risk. An empirical approach is needed to generate mechanistic and causal conclusions.
- Existing *in vitro* approaches are limited in their applicability to the study of foreskin-microbe interactions (Figure 2).[6,7]
- Our *in vitro* organotypic foreskin model utilizes primary human fibroblasts and keratinocytes to reconstruct skin equivalents with a dermis and fully stratified epidermis.
- Organotypic tissue provides a stable, physiologically relevant, reproducible, and highly controllable environment for investigation of foreskin immune responses to specific microbial stimuli.

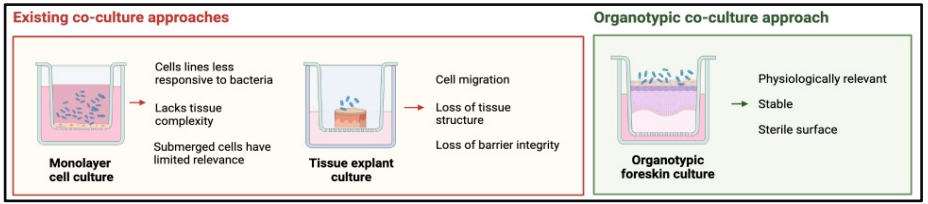

Figure 2. Comparison of existing approaches and organotypic co-culture approach

Objective: Investigate the validity of organotypic foreskin tissue as a model for study of the penile microbiome and its effects on HIV susceptibility, based on:

1. Recapitulation of skin barrier function, which is critical in protection against pathogens, such as HIV.
2. Ability of tissue to support growth of skin commensal, *Staphylococcus epidermidis.*

Methods

Organotypic foreskin culture:

- Primary human fibroblasts and keratinocytes are isolated from donor foreskin tissues.
- Cells are expanded and keratinocytes are immortalized using a vector expressing E6/E7 proteins from HPV-16.
- Fibroblasts (P3-7) are suspended in a bovine type I collagen matrix and cast into transwells. Fibroblasts produce ECM and remodel the matrix, forming a dermal equivalent (7 days).
- Keratinocytes (P3-6) are seeded onto apical surface of the dermal equivalent, expanding to a confluent monolayer in submerged culture (10 days).
- Keratinocytes are exposed to the air-liquid interface, inducing stratification and differentiation of keratinocytes to generate a mature epithelium suitable for co-culture with bacteria.

Barrier Integrity Functional Analysis:

- Dextran tracer exclusion assay assessed barrier function of explant and organotypic tissues via fluorescently labelled dextran tracer molecule (10 kDa), which cannot pass through skin with a healthy, intact barrier.
- If barrier function is low, dextran will permeate the tissue, entering the basal media.
- Dextran tracer was added apically to the tissue surface and basal media samples were taken at 6-,12-, and 24-hour time points. Media fluorescence was measured to determine tissue permeability.

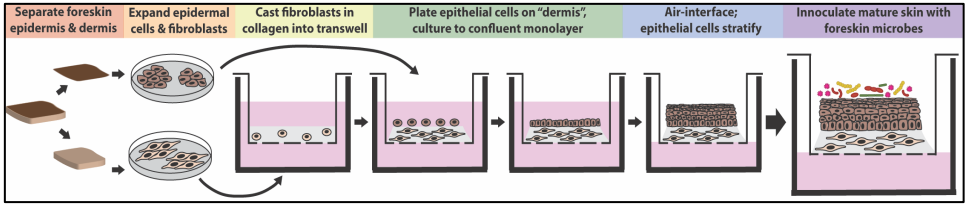

Figure 3. Workflow for generation of organotypic foreskin tissues from primary foreskin fibroblasts and keratinocytes.

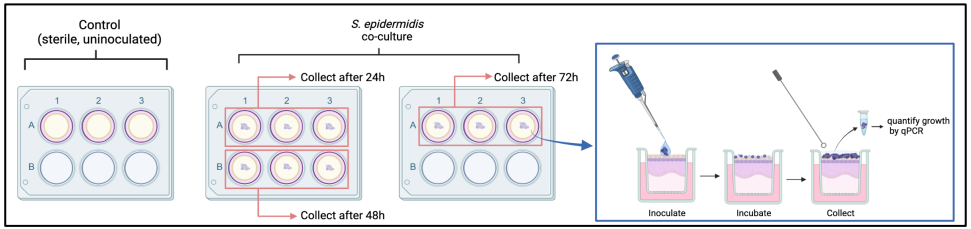

Figure 4. Organotypic foreskin-*S. epidermidis* co-culture design. Bacterial inoculum was added to the tissue surface, co-cultures were then incubated at 37℃ for 72 h. At 24, 36, and 72 h bacteria were collected from the surface of undisturbed tissues (n=3) for quantification of growth by qPCR. Controls were sterile, uninoculated tissues from the same batch (n=3).

Generation of foreskin-bacteria co-cultures:

- Tissue surface washed with PBS prior to inoculation to allow for even distribution of inoculum across the epithelium surface.
- 10 µL inoculum of *Staphylococcus epidermidis* (overnight culture suspended in PBS at ~1500 CFU) added to apical surface of 9 organotypic tissues and incubated at 37℃.
- At 24/48/72-hour timepoints, bacteria were collected from the surface of the tissues (n=3) by scraping with a sterile loop and washing with 100 µL PBS.
- DNA was extracted and quantification of *S. epidermidis* was achieved by qPCR.

Results

Comparison of barrier function between fresh, explant, and organotypic foreskin tissue

- 7-day explant tissues are highly permissive to dextran tracer molecule, showing 59.6 ± 20.8% permeation after 24 h.
- Organotypic tissues have low permeability to dextran tracer, with 3.66 ± 2.59% permeation after 24 h.
- Organotypic tissue shows stable barrier function over a 24 h period, with less variability between replicates, relative to both fresh and 7-day explant tissues.

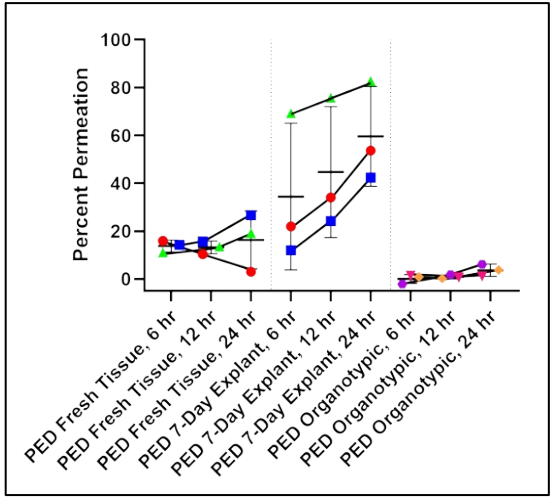

Figure 5. Percent permeation by 10 kDa fluorescent dextran applied to tissue surface for fresh (n=3), 7-day explant (n=3), and organotypic (n=3) tissues. Each point represents tissue from a single donor assayed in triplicate; matched donors are indicated by same point colouration across groupings. Percent permeation calculated by normalization of raw fluorescence to a time-matched positive control (basal media spiked with 10uL dextran).

Figure 6. A mature organotypic tissue after 3 weeks of air-liquid interface culture.

Mature tissues have a dry apical surface, indicating formation of a physical barrier to control water loss.

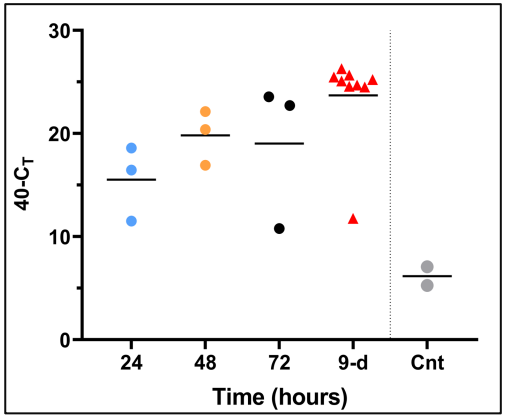

Figure 7. Quantification of *S. epidermidis* growth on organotypic foreskin surface over time. Organotypic tissues grown from a single donor were selected for co-culture with *S. epidermidis* (n=9) or as negative controls (n=3). Bacteria colonizing the tissue surface were collected from undisturbed tissues after 24-,48-, 72- hours of co-culture at 37°C (n=3 for each timepoint). Bacterial DNA was extracted, and growth was determined via qPCR with *S. epidermidis* specific primers. To test growth over an extended period, a final bacterial collection was performed after 9 days of co-culture (n=9).

S. epidermidis growth on organotypic tissue surface

- Trend shows increasing load of *S. epidermidis* after inoculation of the apical surface of organotypic tissues with overnight culture re-suspended in PBS (~1500 CFU) and incubation at 37°C.
- Increased abundance at Day 9 suggests this model may be capable of stably supporting bacterial growth over several days.
- Organotypic tissue remained intact without visible degradation when co-cultured with *S. epidermidis.*

Conclusions

- High permeability of explant cultured tissues (increasing over a 24h period) indicates poor barrier function. Lack of barrier integrity in explant tissues indicates that this approach is unsuitable for study of the penile microbiome and its effects on HIV susceptibility. This further emphasizes the need for a novel *in vitro* approach.

- In contrast, organotypic tissues consistently show low permeability over 24h. Dextran was effectively excluded from organotypic skin, mimicking the expected response of skin *in vivo.* These findings suggest that organotypic tissue has a highly functional barrier, recapitulating an essential property of skin and filling a current gap in the literature.

- Organotypic tissue supports colonization by *S. epidermidis*, a major component of the normal skin microbiota. These findings suggest that organotypic foreskin tissue is capable of co-culture with skin microbes, providing proof of concept for a foreskin-microbe co-culture model and paving the way for future experiments with penile anaerobes relevant to HIV risk.

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Figures 1,2 and 4 created with BioRender.com

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